

Reduction of *E. coli* O157:H7 populations in sheep by supplementation of an experimental sodium chlorate product[☆]

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Abstract

Ruminant animals are naturally infected with the pathogen *Escherichia coli* O157:H7, annually responsible for numerous meat recalls, foodborne illnesses and deaths. *E. coli* are equipped with the enzyme nitrate reductase, which not only enables this bacteria to respire anaerobically, but also converts chlorate to the toxic metabolite chlorite. This enzyme system is particular to only a few intestinal bacteria, therefore the vast majority are not affected by chlorate. Sodium chlorate has been shown to effectively decrease foodborne pathogens in several livestock species, including ruminants. However, because infection and proliferation of *E. coli* occurs primarily in the lower intestine, there is interest in “by-passing” the rumen, thereby, delivering chlorate directly to the largest population of pathogens. The objective of the current study was to evaluate the ability of an experimental sodium chlorate product (ECP II), designed to by-pass the rumen, in reducing fecal shedding and gut concentrations of *E. coli* O157:H7. Twenty crossbred mature ewes were adapted to a high grain ration and experimentally inoculated with *E. coli* O157:H7. Thirty-six hours following inoculation, sheep received in their feed one of the following ECP treatments: (1) control (CON), no chlorate; (2) 1X (LOW); (3) 2X (MED); and (4) 4X (HIGH) where $X = 1.1$ g chlorate ion equivalents/kg BW (five sheep per treatment). Fecal samples were collected every 12 h following inoculation and 24 h following the feeding of chlorate, all animals were euthanized and tissue samples and their respective contents collected from the rumen, cecum and rectum. The MED and HIGH chlorate treatments significantly reduced fecal shedding of *E. coli* O157:H7 compared to the CON treatment [1.53, 1.11, and 3.89 CFU/g feces (\log_{10}), respectively]. Ruminal contents were similar among treatments, while chlorate tended to decrease ($P = 0.08$) and reduced ($P < 0.05$) *E. coli* O157:H7 populations in the cecum and rectum, respectively. Populations of generic *E. coli* in the cecal contents were numerically lower ($P = 0.11$) in the LOW treatment and tended to decrease ($P = 0.06$) in the MED and HIGH chlorate treatments, respectively. Fermentation profiles through the gastrointestinal tract were unaffected as indicated by slight, but not significant, changes in volatile fatty acids (VFA) profiles in sheep fed chlorate. Results from this study indicate that this experimental chlorate product, administered in the feed, was effective in reducing *E. coli* O157:H7 from the lower gut of sheep as evidenced by the lower cecal and rectal but not ruminal concentrations. Feeding chlorate may be an effective method to decrease *E. coli* O157:H7 populations in ruminant animals prior to slaughter. Published by Elsevier Science B.V.

Keywords: *Escherichia coli* O157:H7; Chlorate; Sheep

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1. Introduction

Escherichia coli O157:H7 causes approximately 73,000 illnesses and approximately 60 deaths in the United States each year (Mead et al., 1999) at a cost of nearly US\$ 1 billion (USDA:ERS, 2001). Although outbreaks of *E. coli* in humans have been linked to water, fruit juice, vegetables, venison, and animal contact, the majority of human cases have resulted from foods originating from cattle, usually ground beef, or contamination from cattle manure (Besser et al., 1993; USDA:APHIS, 1997; Gage, 2001). Cattle, sheep, and goats are natural reservoirs for this bacterium (Chapman et al., 1997; Kudva et al., 1997a,b; Chapman, 2000; Pritchard et al., 2000) and typically appear non-symptomatic while shedding this pathogen into the environment (Gansheroff and O'Brien, 2000). Recent advances in the sensitivity of culture techniques to detect *E. coli* O157:H7 have indicated the incidence is much higher than initially reported. Elder et al. (2000) reported an approximate prevalence of 28% in beef cattle in the United States and Zschock et al. (2000) reported approximately 20% of feedlot cattle in Europe are carriers of *E. coli* O157:H7. Post-slaughter sanitation methods effectively reduce carcass contamination with *E. coli* O157:H7 (Elder et al., 2000), however large-scale recalls of ground beef indicate the need for further intervention.

Recently, chlorate supplementation has been investigated as a pre-harvest strategy to reduce populations of *E. coli* O157:H7 and *Salmonella* in food animals (Anderson et al., 2001a,b). Certain bacteria can respire anaerobically by reducing nitrate to nitrite via the intracellular enzyme nitrate reductase (Alaboudi, 1982; Ingledew and Poole, 1984). However, this same enzyme also reduces chlorate to chlorite, a cytotoxic end-product (Stewart, 1988). Chlorate significantly reduced *E. coli* O157:H7 populations in ruminal fluid incubations (Anderson et al., 2000; Callaway et al., 2001), wild-type *E. coli*, inoculated *E. coli* O157:H7 (Callaway et al., 2002), and total coliforms in cattle (Anderson et al., 2002) and inoculated *E. coli* O157:H7 in sheep (Callaway et al., 2003). However, the complexity of the rumen environment and the availability of chlorate in the rumen, may limit its success in killing pathogenic bacteria in the targeted lower intestine. Callaway et al. (2002) reported chlo-

rate was more effective at reducing populations of *E. coli* O157:H7 in the rumen versus the lower intestinal tract. Increasing the amount of chlorate by-passing the rumen, would theoretically increase the effectiveness of chlorate in reducing populations of *E. coli* in the lower gut. The objectives of this study were to: (1) evaluate supplementation of an experimental sodium chlorate product (ECP II), designed to by-pass the rumen on gut and fecal populations of *E. coli* O157:H7 in experimentally infected sheep; and (2) to evaluate if feeding chlorate is an effective method of administration.

2. Materials and methods

2.1. Animals and experimental design

Twenty crossbred (Suffolk × Rambouillet) ewes (average BW = 55 kg) were housed in environmentally controlled facilities (five per pen) and adapted to a 70:30 concentrate (commercial lamb pellet; Table 1) to forage (bermudagrass hay) ration over a 2 week period. Following the adaptation period, ewes were randomly assigned to individual pens and treatments and continued on the above ration with feed and water available for ad libitum consumption. Fecal samples were collected 3 days prior to inoculation and screened for the presence of *E. coli* O157:H7.

The experimental protocol is presented in Fig. 1. All ewes were experimentally inoculated with 10 ml of tryptic soy broth (TSB) containing 2×10^9 CFU/ml *E. coli* O157:H7 via oral gavage at the initiation of the experiment. Fecal samples (approximately 10 g) were collected every 12 h following inoculation for bacterial enumeration described later. Five sheep were

Table 1
Composition of sheep feed (as-fed basis)

Crude protein (%)	15.0
Crude fat (%)	2.50
Crude fiber (%)	14.50
Calcium (%)	0.80
Phosphorus (%)	0.40
Salt (%)	0.25
Copper (ppm)	8.0
Selenium (ppm)	0.3
Vitamin A (IU/lb)	15000

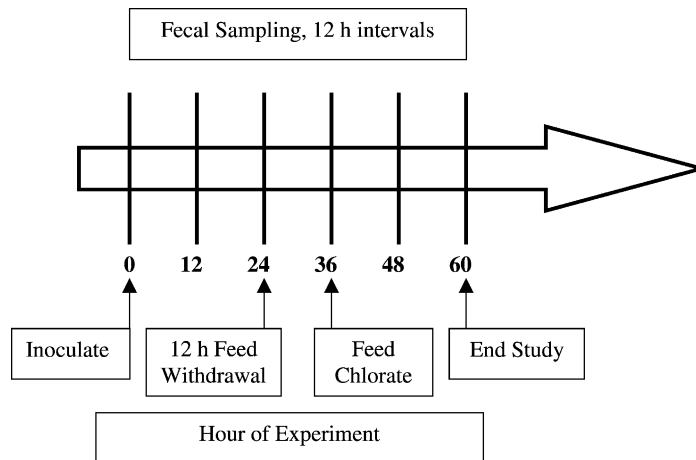


Fig. 1. Experimental protocol.

randomly assigned to each of the following ECP II treatments: (1) control (CON), no chlorate; (2) 1X (LOW); (3) 2X (MED); and (4) 4X (HIGH) where $X = 1.1$ g chlorate ion equivalents/kg BW (five sheep per treatment). The ECP II (EKA Chemicals, Inc., Marietta, GA) was mixed with one-half pound of ground corn and included in the daily feed ration following a 12 h feed withdrawal. Twenty-four hours following chlorate feeding (60 h post-inoculation), sheep were euthanized (Euthasol[®], euthanasia solution, Delmarva Laboratories, Inc., Midlothian, VA) and tissues from the rumen, cecum, and rectum as well as their respective lumen contents were aseptically collected for bacterial enumeration described below. Care, use, and handling of experimental animals was pre-approved by the Animal Care and Use Committee of the Food and Feed Safety Research Laboratory, USDA.

2.2. Bacterial cultures and enumeration

Escherichia coli O157:H7 strain 2336 was obtained from the Field Disease Isolation Unit, Pullman, WA. This strain was made resistant to rifampicin in our laboratory via successive cultivation in TSB containing 20 µg/ml rifampicin. This rifampicin resistant phenotype was stable through multiple unselected transfers in batch culture (data not shown). Overnight cultures (1000 ml) were harvested by centrifugation (7500 × g, 10 min) and the cell pellets were re-suspended in TSB medium (150 ml total volume) for inoculation.

One gram of fecal material was homogenized and serially diluted (10-fold increments) in sterile PBS and plated on MacConkey agar supplemented with 20 µg/ml rifampicin to enumerate inoculated *E. coli* O157:H7. Plates were incubated 24 h at 37 °C and colonies that grew on agar plates directly counted. To qualitatively confirm the presence of inoculated *E. coli* O157:H7 and generic *E. coli*, luminal contents and epithelial tissue samples were incubated (24 h, 37 °C) in 20 ml GN Hajna with rifampicin and streaked on MacConkey agar supplemented with 20 µg/ml rifampicin (for inoculated *E. coli* O157:H7) and M-Endo agar LES (generic *E. coli*). Plates showing characteristic colony growth after 24 h incubation were judged to be positive (qualitative enumeration).

Intestinal contents were analyzed for volatile fatty acids (VFA) concentrations as previously described (Corrier et al., 1990). Unless otherwise noted, all media and agar were from Difco Laboratories, Detroit, MI. Reagents were obtained from Sigma Chemical Co., St. Louis, MO.

2.3. Statistical analysis

Data were analyzed using SAS Version 8.02 (SAS Inst. Inc., Cary, NC). Data for fecal shedding of bacteria were analyzed using the Proc Mixed procedure with treatment, hour and ewe included in the model and reported as least square means ± S.E.M. Logistic regression was used to analyze

qualitative bacterial enumeration. Bacterial counts for luminal contents (quantitative) were subjected to analysis of variance appropriate for a completely randomized design.

3. Results

Prior to inoculation with *E. coli* O157:H7, sheep did not shed *E. coli* populations capable of growth on rifampicin supplemented MacConkey agar (data not shown). Fecal shedding of *E. coli* O157:H7 throughout the experimental period is shown in Fig. 2. Populations of *E. coli* O157:H7 ranged from 10^4 to 10^6 CFU/g of feces through 48 h following inoculation with no differences prior to chlorate treatment. There was a significant treatment \times time interaction ($P = 0.01$) related to chlorate administration. No differences in fecal shedding of *E. coli* O157:H7 were observed ($P > 0.10$) 12 h following chlorate treatment. However, 24 h following chlorate feeding, *E. coli* O157:H7 populations were decreased ($P < 0.05$) two logs in the MED and HIGH chlorate treatments when compared to CON animals. The LOW chlorate treatment was one log lower than the CON treatment however this was not significantly different.

Populations of *E. coli* O157:H7 isolated from luminal contents of the rumen, cecum and rectum are presented in Fig. 3. No differences ($P > 0.10$) were observed in *E. coli* O157:H7 in the rumen contents, with low counts (approximately one log) in all treatments. Chlorate tended ($P = 0.08$) to decrease *E. coli* O157:H7 in cecal contents, particularly the HIGH treatment with a two log reduction. Inoculated *E. coli* O157:H7 populations in the rectal contents were decreased ($P = 0.04$) by the MED and HIGH doses of chlorate and numerically lower in the LOW chlorate treatment. Tissue samples from the rumen, cecum and rectum, after enrichment, were not different ($P > 0.10$) among treatments, with the majority of samples positive for *E. coli* O157:H7 (data not shown).

Populations of generic *E. coli* were similar to those of *E. coli* O157:H7 in luminal contents from the rumen, rectum and cecum (Fig. 4). Ruminal populations of generic *E. coli* were low with no differences ($P > 0.20$) observed among treatments. Cecal contents were numerically lower ($P = 0.11$), particularly in the HIGH treatment when compared to controls (1.88 versus 4.54 CFU/g \log_{10}). Chlorate tended ($P = 0.06$) to decrease generic *E. coli* counts in rectal contents, exhibiting an almost linear response.

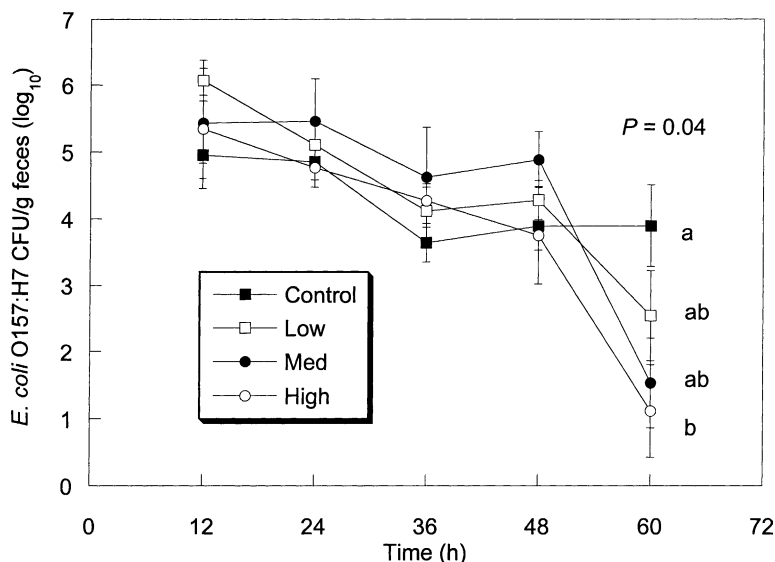


Fig. 2. Fecal shedding of *E. coli* O157:H7 (CFU/g feces \log_{10}) after experimental infection (at time = 0) in sheep fed 0 (CON), 1X (LOW), 2X (MED) or 4X (HIGH; $X = 1.1$ g chlorate ion equivalents/kg BW) of an experimental sodium chlorate product 24 h prior to slaughter. (a, b) Different letters by symbols indicate that, within time, treatment means differ ($P < 0.05$).

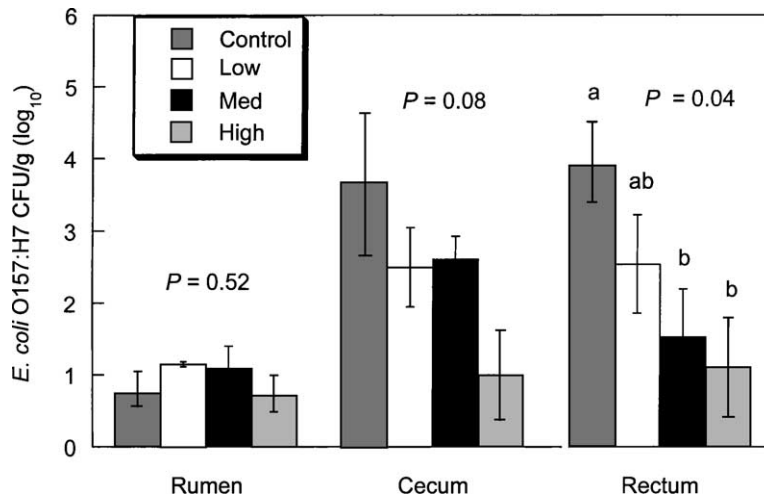


Fig. 3. Populations of *E. coli* O157:H7 from the luminal contents of the rumen, cecum and rectum in experimentally infected sheep fed 0 (CON), 1X (LOW), 2X (MED) or 4X (HIGH; X = 1.1 g chlorate ion equivalents/kg BW) of an experimental sodium chlorate product 24 h prior to slaughter. (a, b) Different letters above bars indicate that, within tissue type, treatment means differ ($P < 0.05$).

Chlorate treatment did not significantly impact the fermentation profile throughout the gastrointestinal tract as indicated by VFA analysis presented in Table 2. In some instances, chlorate tended ($P < 0.10$) to lower VFA concentrations in the lower gut samples.

4. Discussion

Escherichia coli O157:H7 infections in humans cause hemorrhagic colitis that can progress to the life-threatening hemolytic-uremic syndrome (Blaser et al., 1995). The majority of these cases have been

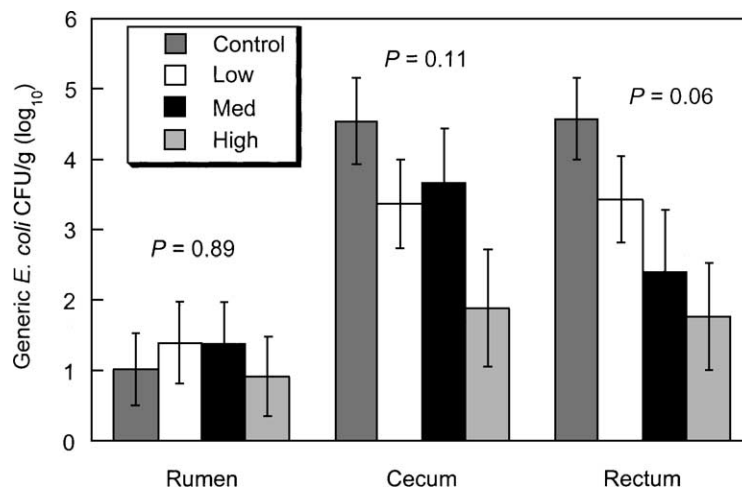


Fig. 4. Populations of generic *E. coli* from the luminal contents of the rumen, cecum and rectum in sheep experimentally infected with *E. coli* O157:H7 and fed 0 (CON), 1X (LOW), 2X (MED) or 4X (HIGH; X = 1.1 g chlorate ion equivalents/kg BW) of an experimental sodium chlorate product 24 h prior to slaughter.

Table 2

Volatile fatty acids (mM) from the rumen, cecum and rectum of sheep fed 0 (CON), 1X (LOW), 2X (MED) or 4X (HIGH; where X = 1.1 g chlorate ion equivalents/kg BW) of an experimental sodium chlorate product 24 h prior to slaughter

Item		Chlorate treatment ^a				S.E.M.	P-value
		CON ^b	LOW	MED	HIGH		
Rumen	Acetate	39.2	36.03	29.57	28.7	3.89	0.2
	Propionate	14.07	13.71	18.04	16.03	2.25	0.51
	Total	60.05	55.85	50.65	48.08	6.57	0.58
Cecum	Acetate	58.91	64.83	65.68	48.28	4.86	0.08
	Propionate	17.92	17.75	19.06	13.7	1.64	0.15
	Total	79.01	85.34	87.18	62.69	6.54	0.07
Rectum	Acetate	57.1	36.76	51.37	40.67	5.98	0.09
	Propionate	17.12	10.26	15.75	11.89	2.03	0.09
	Total	77.11	47.63	68.82	53.3	8.47	0.08

^a Five ewes per treatment.

^b CON: control.

linked to the ingestion of contaminated bovine products, most often undercooked ground beef (Kaper and O'Brien, 1998; Gage, 2001).

Sheep, like cattle, are naturally colonized by *E. coli* O157:H7 in a transient and seasonal manner (Chapman et al., 1996; Kudva et al., 1996, 1997a,b; Johnsen et al., 2001) and have been documented to have populations of *E. coli* O157:H7 similar to those found in cattle (Zschock et al., 2000). However, the number of human outbreaks of *E. coli* that are attributed to ovine rather than bovine sources are far less. Sheep serve as an effective and economical model of *E. coli* O157:H7 colonization and infection in ruminants (Kudva et al., 1995, 1997a,b; Cookson et al., 2001; Cornick et al., 2000; Wales et al., 2000).

Recently, 19 million pounds of beef and trimmings was recalled after being linked to several illnesses caused by *E. coli* O157:H7 in the United States (Food Safety Report, 2002). Although numerous post-harvest intervention methods were in place and have been shown to successfully reduce carcass contamination (Elder et al., 2000), recalls like the one above indicate that these methods are by no means 100% effective. Strategies that reduce specific food-borne pathogens entering the abattoir could produce “the most significant reductions in human exposures to the organism and therefore in related illnesses and deaths” (Hynes and Wachsmuth, 2000).

Researchers at our laboratory have recently investigated the use of sodium chlorate to reduce *E. coli* O157:H7 and *Salmonella* populations in food

animals (Anderson et al., 2001a,b). These bacteria can respire anaerobically by reducing nitrate to nitrite via a dissimilatory nitrate reductase (Ingledew and Poole, 1984; Stewart, 1988). This same system also reduces chlorate to chlorite, a toxic compound to *E. coli* and *Salmonella*. Early work with ruminants found the addition of chlorate significantly reduced *E. coli* O157:H7 in ruminal fluid incubations (Anderson et al., 2000; Callaway et al., 2001) and wild-type *E. coli* populations in cattle when administered directly to the rumen (Anderson et al., 2002). Chlorate administered in the drinking water of cattle significantly reduced populations of three strains of inoculated O157:H7, total coliforms and wild-type *E. coli* (Callaway et al., 2002). Recent research reported similar results in sheep, noting a significant reduction in inoculated *E. coli* O157:H7, generic *E. coli*, and total coliforms (Callaway et al., 2003).

Ruminant nutritionists have recognized that in certain cases (e.g. proteins, fat) efficiency of nutrient utilization and animal performance may be increased if the rumen can be “by-passed” and the lower gastro-intestinal tract reached. The ability of the ruminal microflora to utilize chlorate may be limiting the effectiveness of chlorate administration in cattle and sheep. The present study examined the effects of feeding an experimental sodium chlorate compound, with rumen by-pass characteristics, prior to slaughter on populations of experimentally inoculated *E. coli* O157:H7 and generic *E. coli* in sheep. Our results showed that ECP II decreased *E. coli* O157:H7 and

generic *E. coli* populations in sheep, findings similar to other chlorate studies in ruminants (Callaway et al., 2003; Anderson et al., 2002). The success of by-passing the rumen in this experiment is unclear. Populations of *E. coli* were significantly reduced in the lower gut, but not the rumen, which suggests the chlorate product was more effective in the lower gut. Similar research conducted in our laboratory (Callaway et al., 2003) using a different chlorate product with no rumen by-pass characteristics, found chlorate significantly decreased rumen as well as hind-gut concentrations of inoculated *E. coli* O157:H7. This also supports findings in the current study, that the lack of an effect on *E. coli* O157:H7 populations in the rumen, may be due to the ECPII by-passing the rumen as expected. However, *E. coli* populations were low in the rumen to begin with and therefore any effect may have been difficult to detect based on animal numbers or assay sensitivity at these low populations. This is consistent with previous findings that *E. coli* is found in the rumen and upper GIT (Cray and Moon, 1995) however, the proportion of *E. coli* relative to total bacterial flora increases in the lower GIT. *E. coli* O157:H7 and generic *E. coli* reside primarily in the hindgut in both sheep and cattle (Dean-Nystrom et al., 1999; Buchko et al., 2000; Grauke et al., 2002). In contrast, others have reported that the rumen is the primary site of *E. coli* O157:H7 colonization (Brown et al., 1997; Zhao et al., 1998) although these studies were done with young (8–11-week-old) calves that may not have had a fully developed ruminal microbiota. Previous research generally agrees that the lower GIT is the primary site for persistence and proliferation of *E. coli* O157:H7 and supports the need for a chlorate product that by-passes the rumen.

As with any kind of treatment, determining the most effective delivery method is crucial for success. Previous research (Callaway et al., 2002, 2003) administered chlorate in the drinking water of ruminants and found this to be an effective means of delivery. However, administration in the water may not always be the most viable alternative, therefore the current study examined the effectiveness of incorporating chlorate in a single feeding. All the sheep consumed the chlorate-containing feed within 1 h, and although it appeared to slow intakes compared to the controls, it is important to recognize that the chlorate was administered in a small portion of the daily feed to

ensure intake. These same chlorate concentrations incorporated into the full daily feed allotment would probably not have affected intakes. Although the reduction in pathogen levels we observed were not of the same magnitude as reported previously (Callaway et al., 2003) we did demonstrate that administration of chlorate in the feed may be a viable delivery option.

While not all intestinal bacteria are sensitive to chlorate, some important bacterial species (e.g. *Selenomonas*, *Wolinella*) are equipped with nitrate reductase and are theoretically susceptible to chlorate. Previous work demonstrated that the addition of chlorate did not alter gastrointestinal VFA profiles in cattle or sheep (Callaway et al., 2002 a,b). In the current study, ECP II tended to alter VFA profiles, however, these differences were not significant and would have little to no effect on animal performance or well being if administered 24–48 h prior to slaughter.

5. Conclusion

Modern post-harvest intervention strategies are effective in reducing carcass contamination in the processing facilities following slaughter. However, pathogenic bacteria still enter the food chain causing economic losses, human illness and in severe cases, death. In the present study, an experimental sodium chlorate product reduced *E. coli* O157:H7 populations in the feces and luminal contents from the cecum and rectum. Elder et al. (2000) demonstrated a direct correlation between fecal populations of *E. coli* O157:H7 and the level of carcass contamination. This indicates that chlorate treatment within 24 h of slaughter could decrease possible carcass contamination by reducing fecal populations of foodborne pathogens in the animal. Further research is needed to determine the most effective treatment methodologies. Incorporating knowledge from this work and others along with pre- and post-harvest intervention strategies may help to improve the safety of ruminant derived foods.

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